CHROMATOGRAPHY OF QUATERNARY NITROGEN COMPOUNDS ON BUFFERED CATION-EXCHANGE RESINS

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A procedure for the separation of betaines and other quaternary nitrogen compounds on columns of sulfonated polystyrene resin with acid eluents has been described by CHRISTIANSON *et al.*[1]. This method was especially satisfactory for isolating and characterizing many compounds from biological extracts because of easy removal of the eluting agent, dilute hydrochloric acid. However, certain compounds like amides or esters were decomposed by the acid, and others were not well separated in the acid system. Buffered columns as employed by MOORE, SPACKMAN AND STEIN[2] were investigated as a means of eliminating these disadvantages. An improvement in chromatography of amino acids on buffered columns has been made by HAMILTON[3] through the use of very fine cation-exchange resins of selected uniform particle size. Sharp resolution was maintained at accelerated flow rates obtained under pressure, thereby reducing time of chromatography.

This report describes the adaptation of these buffered column procedures to chromatographic separation of some betaines and other naturally occurring quaternary nitrogen compounds. These compounds were detected and determined by a modification of the procedure of WALL *et al.*[4] based on ultraviolet absorbance of their periodide derivatives. More rapid and better resolution of mixtures containing betaine, choline, carnitine, trigonelline, stachydrine, and others was achieved with this system than with columns in the hydrogen form[1]. For further characterization, the quaternary nitrogen compounds were isolated from the buffer effluent as their periodide derivatives, which were then converted to the original compound.

An application of the technique to the analysis of quaternary nitrogen compounds in extracts of whole corn is described.

METHODS

Buffers and ion-exchange columns

Sodium citrate buffers, pH 2.2, 3.25, and 4.25 containing 0.20 *N* sodium ion, and pH 5.28 containing 0.35 *N* sodium ion, were prepared as described by MOORE, SPACKMAN AND STEIN[2] except that BRIJ 35 detergent and phenol were omitted. The addition of detergent resulted in anomalous yields of periodide precipitates, and phenol interfered with measurements of the ultraviolet absorption of the effluent.

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A finely ground sulfonated polystyrene resin, Amberlite IR-120 (CG-120) type 2 (Rohm & Haas, Co., Philadelphia, Pa.)*, was separated into more uniform particle size fractions by the method of HAMILTON[3]. More recently preparations of the resin with selected particle sizes suitable for chromatography were purchased from Bio-Rad Laboratories, Berkeley, Calif. (Aminex-MS, fractions C and D.). The 0.9-cm diameter columns of these resins were packed following the procedure of MOORE *et al.*[2] employing fraction C for 15-cm and 50-cm height columns and D for 150-cm columns. After being washed with 0.2 *N* NaOH they were equilibrated with the pH 3.25 buffer.

Chromatography of known compounds

Mixtures of known quaternary nitrogen compounds containing 0.5–2.0 mg of each substance were dissolved in the pH 2.2 buffer and applied to 15-cm, 50-cm, and 150-cm long columns, 0.9 cm in diameter, jacketed for temperature control. The solutions also included 0.5–1.0 μmole of amines and amino acids related to the quaternary nitrogen compounds to enable a comparison of their behavior during chromatography. The compounds tested were high-purity products obtained from commercial sources or synthesized as described previously[4]. Initial elution usually was with the pH 3.25 buffer at 30° followed by buffers of higher pH with operation at elevated temperatures to remove compounds held more tenaciously. The columns were operated under pressures of nitrogen that gave the flow rates, 12–15 ml/h, recommended by MOORE *et al.*[2].

Corn extract

The corn extract subjected to chromatography was obtained by 80 % ethanol extraction of ground dent corn, a composite of commercial varieties. Details of preparation of sample, extraction procedure, and removal of contaminants, including proteins and lipids from the extract, are described elsewhere[5]. The final aqueous extract was analyzed for total nitrogen by the Kjeldahl method. A portion was taken to dryness and made up to volume with pH 2.2 buffer solution so that a 2-ml aliquot contained a suitable sample for chromatography.

Determination of quaternary nitrogen compounds

Column effluent fractions were analyzed for quaternary nitrogen compounds by measurement of the ultraviolet absorbance of their periodide derivatives. The reagent used for the preparation of the periodides in the buffered solutions consisted of 15.7 g iodine and 20.0 g potassium iodide dissolved in 100 ml of 3 *N* sulfuric acid. The sulfuric acid was added to reduce the pH of the buffered effluent fractions to a value giving optimum yields of periodide precipitates. Because iodine precipitated from the reagent upon standing, fresh reagent was prepared daily. To 0.5-ml aliquots of effluent fractions or standard solutions containing 10–100 μg of quaternary nitrogen compound in appropriate buffers, 0.2 ml reagent was added and the periodide obtained as described by WALL *et al.*[4]. The absorbance of the periodide dissolved in 5.0 or 10.0 ml ethylene dichloride was measured. Standard curves or equations relating optical density and periodide yields were used to determine the amounts of quaternary nitrogen compounds in unknown samples[4]. Values of correction factors for solubility

* Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

losses and of absorbances of periodides prepared from buffered solutions of quaternary nitrogen compounds are listed in Table I.

Initial survey of the effluent fractions was made by analyzing every third tube. Each fraction in the vicinity of a periodide-yielding tube was then reanalyzed care-

TABLE I

FACTORS FOR ESTIMATING QUATERNARY NITROGEN COMPOUNDS FROM PERIODIDE ABSORPTION *

Quaternary nitrogen compound	*Original pH of elution buffer*	*Periodide solubility (μg original compound in 0.7 ml reaction solution)*	*μg of quaternary nitrogen compound per unit absorbance of periodide at 365 mμ in 5 ml of ethylene dichloride*
Betaine·HCl	3.25	13.2	25.1
γ-Butyrobetaine·HCl	3.25	0.7	39.3
Carnitine·HCl	3.25	23.2	36.3
Choline chloride	3.25	0.9	27.0
N-Methylnicotinamide	5.28	1.1	40.7
Stachydrine·HCl	3.25	2.9	40.2
Trigonelline·HCl	3.25	2.0	31.2

* Values should be redetermined to allow for any variation in experimental conditions.

fully by using 0.1 to 0.5-ml aliquots made up to 0.5 ml by addition of the appropriate buffer where necessary. When larger aliquots were required, the volume of reagent was increased proportionally as was the correction factor for solubility losses.

Other analytical procedures

The amino acids and amines in effluent fractions were determined by the colorimetric ninhydrin method of MOORE AND STEIN[6]. The aromatic compounds were detected in the effluent fractions by measuring absorbance at 260 mμ with the Beckman D.U. spectrophotometer.

Identification of quaternary nitrogen compounds

The quaternary nitrogen compounds were isolated from the pooled remainders of the effluent buffer solution fractions constituting the peak by forming their water-insoluble periodide derivatives as described for the analytical procedure but on a larger scale. As little as 200 μg of the quaternary nitrogen compound can be converted to the chloride salt in essentially quantitative yields by the method of STANEK[7]. For this conversion the periodide was reacted with 0.5 g powdered copper to reduce the complexed iodine. Then 0.2 g copper chloride and 2 ml water were added to the mixture which was heated 1 h in a boiling water bath. The resulting cuprous iodide was removed by centrifugation. Excess copper was removed by addition of H_2S and filtration. The identity of the quaternary nitrogen compound was verified by comparison with known compounds on paper chromatograms. The solvents and techniques employed to separate and detect the compounds on papers have been summarized by BLOCK *et al.*[8].

RESULTS

Chromatography of known substances

Betaine, trigonelline and choline were well resolved in that sequence on a 15-cm column with 120 ml pH 3.25 sodium citrate buffer at 30° (Fig. 1).

A more complex synthetic mixture required a 50-cm column for resolution with an elution schedule of 300 ml pH 3.25 buffer at 30° followed by 400 ml pH 5.28 buffer

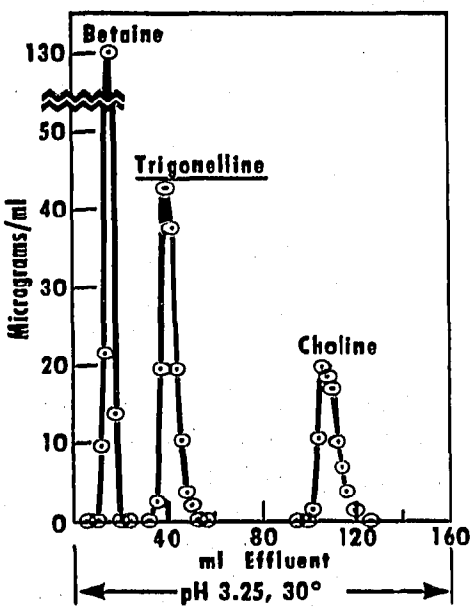

Fig. 1. Separation of betaine, trigonelline, and choline on Aminex-MS, 15-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion.

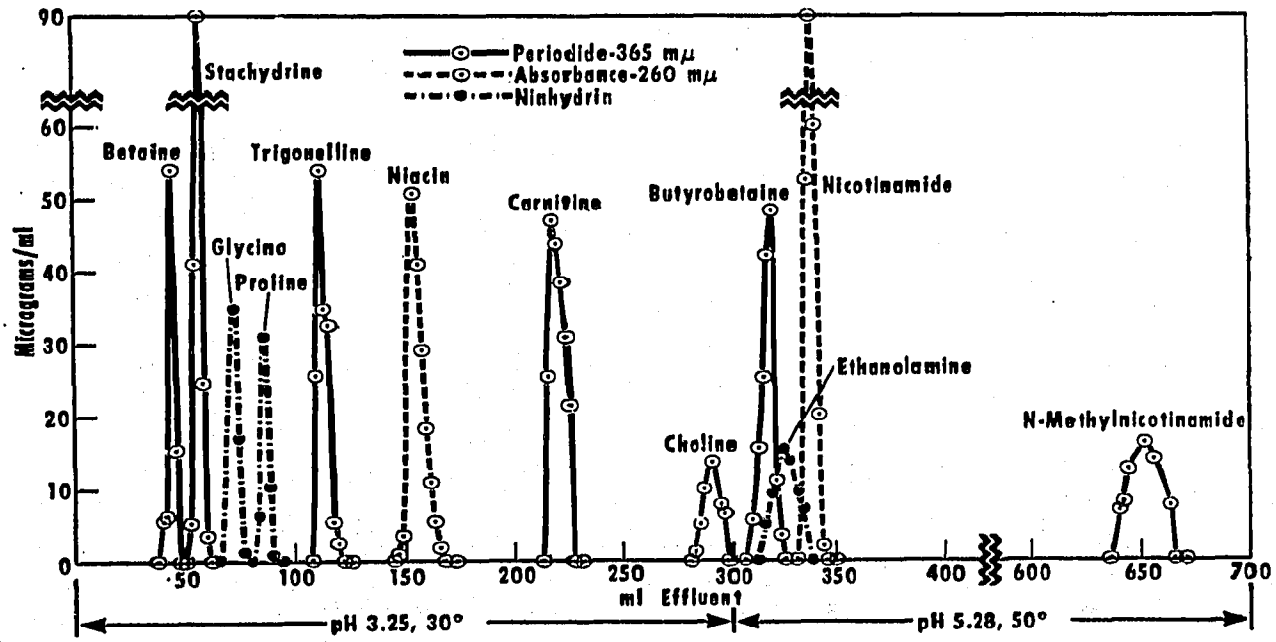

Fig. 2. Separation of a synthetic mixture of quaternary nitrogen compounds and their related amino acids or amines on Aminex-MS, 50-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion and pH 5.28 sodium citrate buffer, 0.35 *N* sodium ion.

at 50°. The elution positions of the quaternary nitrogen compounds and their related amino acids or amines from this column are shown in Fig. 2.

Better separation of earlier eluting quaternary nitrogen compounds was obtained by use of a 150-cm column with 250 ml pH 3.25 buffer. However, the more basic

TABLE II

RECOVERIES OF QUATERNARY NITROGEN COMPOUNDS AFTER ION-EXCHANGE CHROMATOGRAPHY

Substance	*Number of chromatographic determinations*	*Samples applied (mg)*	*Average recovery (%)*	*Average deviation (%)*
Betaine·HCl	3	0.61, 0.61, 1.51	100.8	±2.5
Stachydrine·HCl	3	0.62, 1.22, 1.22	100.3	±3.1
Trigonelline·HCl	3	0.62, 1.22, 1.22	102.2	±1.7
Carnitine·HCl	4	1.20, 1.20, 1.20, 1.51	95.7	±2.5
γ-Butyrobetaine·HCl	2	0.99, 1.14	100.6	±4.3
Choline chloride	2	0.57, 0.63	103.1	±1.5
N-Methylnicotinamide	1	1.50	97.0	

compounds required large volumes of pH 5.28 buffer for elution. Buffer eluent schedules and/or column temperatures can be varied to facilitate separation of specific compounds. For example, γ-butyrobetaine will precede choline on the 50-cm column if, after 200 ml pH 3.25 buffer, elution is continued with pH 4.25 buffer. From a 15-cm column initially equilibrated with pH 5.28 buffer, N-methylnicotinamide emerged after an elution with 200 ml pH 5.28 buffer at 50° in a sharp peak well separated from the other compounds. The more neutral quaternary nitrogen compounds were eluted early with poor resolution similar to the behavior of acidic and neutral amino acids in this system[2].

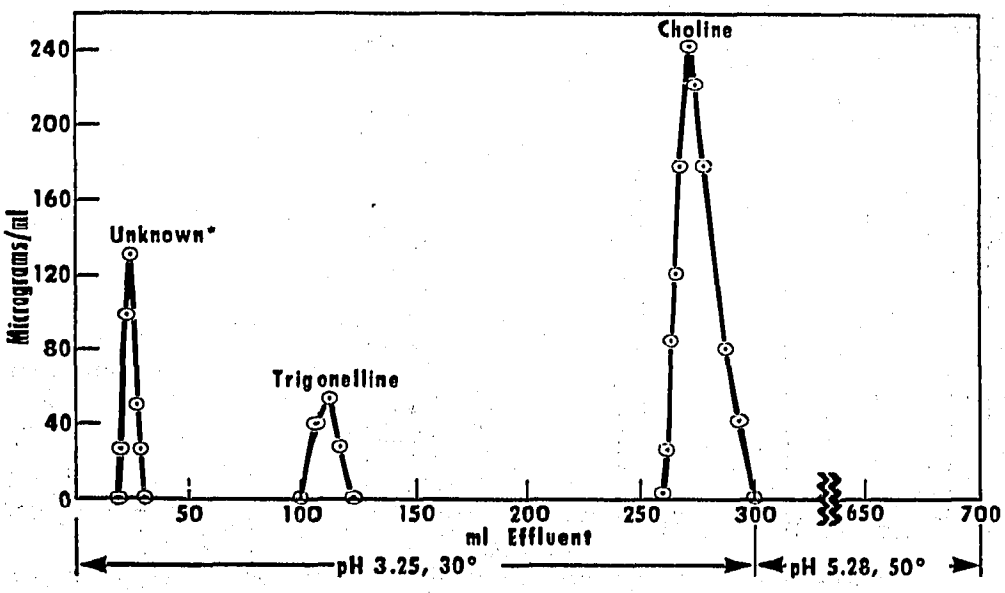

Fig. 3. Chromatographic separation of quaternary nitrogen compounds from whole corn grain on Aminex-MS, 50-cm column, pH 3.25 sodium citrate buffer, 0.20 *N* sodium ion and pH 5.28 sodium citrate buffer, 0.35 *N* sodium ion. (*Microgram unknown based on betaine periodide absorbance).

The recoveries of the compounds separated on a 50-cm column tabulated in Table II were essentially quantitative. The measured recovery of carnitine was low because the high solubility of its periodide may introduce errors in the analysis.

Chromatography of corn extract

An extract equivalent to 30 g corn was chromatographed as illustrated in Fig. 3. Two substances yielding periodide precipitates were identified as trigonelline and choline by position of elution and paper chromatography. These compounds have been chromatographically isolated and identified previously[1] as constituents of corn.

An unknown substance yielding a periodide derivative was also detected in the initial fractions. Work is in progress to characterize this compound.

DISCUSSION

Chromatography on buffered columns offers the same advantages in the separation of quaternary nitrogen compounds as those described for the separation of amino acids by MOORE AND STEIN[9]. As indicated by the recovery values in Table II, the destruction of amides such as N-methylnicotinamide was hardly significant when the buffer procedure was used, whereas the use of hydrochloric acid eluents caused 69 % degradation of this substance[1].

The use of the buffered column resulted in improved separation of certain compounds not well resolved by the acid system, since their differences in charge were emphasized at the pH's of the buffers. For example, betaine and choline were well separated with the buffered columns while with acid columns these substances were eluted in close proximity[1] because of the diminished carboxyl ionization. In the buffered systems the differences in acidity between α- and ω-carboxyls of the betaines resulted in butyrobetaine and carnitine being eluted after trigonelline, similar to the elution of γ-aminobutyric acid after α-aminobutyric acid as reported by SPACKMAN, STEIN AND MOORE[10].

Most of the quaternary nitrogen compounds were eluted before their amine analogues by buffer as indicated by the pairs: glycine and betaine, trigonelline and nicotinic acid, and choline and ethanolamine (Fig. 2). The greater charge on the quaternary nitrogen compounds at the pH of the buffers makes them more polar than the amines or amino acids. Thus quaternization increases the affinity for the polar eluent more than it does adsorption to the aromatic resin. An exception to this is N-methylnicotinamide, which is eluted much after nicotinamide. The quaternary compounds were usually eluted after their related amines on columns wholly in the hydrogen form[1].

The fine mesh-sized resin enabled satisfactory resolution at accelerated flow rates. Thus a typical analysis on the 50-cm column can be completed in 2 days in contrast to the 6 days needed for chromatography on a 200–400 mesh resin. This improvement in rate permits use of the chromatographic method for more routine analysis of plant and other biological extracts. The finer mesh resin of selected size can also be employed for chromatography with acid eluents at accelerated rates where the elution pattern obtained with that system is desired.

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SUMMARY

Quaternary nitroge ncompounds and their amine analogues were separated by chromatography on columns of a uniformly sized, pulverized, sulfonated polystyrene resin using buffers for elution. Substances of biological interest thus separated and determined by the ultraviolet absorption of their periodide derivatives include betaine, stachydrine, choline, trigonelline, N-methylnicotinamide, and carnitine. Better separation of these compounds is obtained with buffered eluents than with hydrochloric acid because of greater differences in degrees of ionization in the buffer system. Corn grain extracts were rapidly analyzed by the procedure.

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